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(54) Title: RECOMBINANT PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV) FOR USE AS A VACCINE

(57) Abstract

The present invention relates to a recombinant Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) vaccine comprising PRRSV structural genes linked to a heterologous polymerase gene and the method for constructing the recombinant virus. Specifically, the recombinant virus comprises the genes encoding open reading frames 1a and 1b from Equine Arteritis Virus (EAV) and the genes encoding open reading frames 2 through 7 from PRRSV. The invention is useful for producing PRRSV vaccines that are resistant to mutaions that currently occur during propagation of PRRSV for vaccines. Furthermore, the invention provides a means for distinguishing swine vaccinated with the aforementioned invention from swine naturally infected with PRRSV.

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RECOMBINANT PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV) FOR USE AS A VACCINE

CROSS REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Patent Application No. 60/048,662, filed June 5, 1997, for Recombinant Porcine Reproductive and Respiratory Syndrome Virus for Use as a Vaccine.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a recombinant Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) comprising a viral polymerase encoding sequence joined to open reading frames (ORFs) from PRRSV. Specifically, the recombinant virus will comprise genes encoding open reading frames 1a and 1b from Equine Arteritis Virus (EAV) and the genes encoding open reading frames 2 through 7 from PRRSV. The present invention also relates to vaccines produced from recombinant PRRSV that are less mutagenic than currently available PRRSV vaccines. The present invention further relates to a method of distinguishing swine vaccinated with the recombinant PRRSV from swine infected with a naturally occurring PRRSV isolates.

Summary of the Related Art

Porcine reproductive and respiratory syndrome virus (PRRSV) is the agent responsible for devastating economic losses to the swine industry. First discovered in 1987, porcine reproductive and respiratory syndrome (PRRS) has rapidly become the most important disease to swine producers worldwide. The disease syndrome has been referred to by many different names such as swine infertility and respiratory syndrome (SIRS), porcine epidemic abortion and respiratory syndrome (PEARS), mystery swine disease, and blue eared pig disease.

The disease agent has been identified as a virus which is a member of the *Arterivirus* genus within the *Togaviridae* family. The PRRSV is a small enveloped spherical positive strand RNA virus, with an average virion diameter of 62 nm and a 25-30 nm core surrounded by an envelope (Goyal, J Vet Diagn Invest Vol. 5 pages 656-664). PRRS is characterized by reproductive failure (abortions), respiratory disease and variable clinical signs that include anorexia, fever, dyspnea, and neurological signs. Macrophages of the lung appear to be the primary type of cell that becomes infected. However, because infection can lead to abortions and neurological dysfunction, the virus seems to be able to spread to other tissues as well. In fact, PRRSV has been isolated from boar semen, placenta, and neurological tissue. Recently, extremely virulent outbreaks of PRRS have been reported in the United States. These outbreaks have resulted in increased abortions and mortality in swine herds.

Multiple strains of PRRSV have been identified, with new strains being routinely reported. Currently, there are two major serotypes of PRRSV, a serotype comprising North American strains and a European serotype comprising the Lelystad virus and related strains (Kapur *et al.*, 1996, J Gen Virol, Vol. 77 pages 1271-1276; Murtaugh *et al.*, 1995, Arch Virol Vol. 140 pages 1451-1460; Meng *et al.*, 1995, Arch Virol Vol. 140 pages 745-755; Katz *et al.*, 1995, Vet Microbiol Vol 44 pages 65-76). Sequence comparison of these two PRRSV serotypes has indicated a nucleotide divergence between the serotypes of about 40%. PRRSV strains within each serotype display considerable nucleotide variation indicating a considerable degree of evolutionary divergence has occurred since emergence of PRRSV. Furthermore, the extent of evolutionary divergence of PRRSV within the short time period since its emergence has indicated the virus is relatively unstable and has not completely adapted to its host.

The PRRSV RNA genome is approximately 15.1 kilobases (kb) in length, polyadenylated and is organized into 8 open reading frames (ORFs). The first 211

nucleotides at the 5' end of the genome, 13 nucleotides between ORFs 1b and 2, and the last 514 nucleotides at the 3' end just upstream of the poly(A) are untranslated. The genome of PRRSV is transcribed into one genome length minus strand RNA transcript that serves as a template for a genome length mRNA, encoding ORFs 1a and 1b, and a 3'-co-terminal nested set of six sub-genomic mRNAs. Each mRNA is polyadenylated and the sub-genomic mRNAs each possess a leader sequence derived from the 5'-end of the viral genome. Each sub-genomic mRNA is presumed to express a single protein encoded by the most forward ORF. (For a general review of PRRSV see Plagemann (Fields Virology, Third Edition (1996), Fields, *et al.* (ed.), Lippincott-Raven Publishers, Philadelphia, PA. pp 1105-1120.)

The 5' end of the genome spans approximately 11 kb and encodes two large proteins, 1a and 1b. ORF 1a protein is 2,397 amino acids and possesses several functional motifs: papain-like cysteine protease and 3C-like serine protease. ORF 1b protein is approximately 1,532 amino acids and possesses replicase, helicase and zinc-finger motifs. An additional motif at the carboxyl terminus of 1b is of unknown function but appears to be highly conserved in the Arteritis genus that includes EAV, lactate dehydrogenase-elevating virus (LDV), and PRRSV, and is also related to a motif found in the polymerase gene of viruses from the coronavirus family. Expression of ORF 1b is via a frameshift mechanism at a site that overlaps ORF 1a and ORF 1b.

ORFs 2 through 7 overlap to varying degrees with their neighbors and the ORFs are read in alternate frames. ORFs 2, 5, and 7 are read in one frame, ORF 4 is read in another frame, and ORF 3 and 6 are read in the third frame. ORF 1b does not overlap with ORF 2. ORF 2 encodes a 250 amino acid minor envelope glycoprotein, ORF 6 encodes a 174 amino acid nonglycosolated envelope protein, and ORF 5 encodes the 202 amino acid primary envelope glycoprotein. ORF 5 gene product may also be involved in virus attachment to the cell and/or internal viral transport and have a role in apoptosis. ORF 7 encodes a 129 amino

acid nucleocapsid protein. ORFs 3 and 4 encode, respectively, 266 and 184 amino acid proteins of unknown function.

Many isolates of PRRSV have been successfully propagated on primary cultures of porcine alveolar macrophages (PAM) (Bautista *et al.*, 1993, J Vet Diagn Invest vol. 5 pages 408-409) and on simian cell lines derived from African green monkey cells such as 9009B (U.S. Patent 5,510,258), MA-104 (U.S. Patent 5,476,778), or MARC-145 (Kim *et al.*, 1993, Arch Virol Vol. 133 pages 477-483). The extent to which any particular isolate replicates to high titers on any of these simian derived cell lines is unique to the specific PRRSV isolate. Since these cell lines are not the natural host for PRRSV, a limited number of passages of PRRSV on these simian derived cell lines results in virus with genomes that have undergone multiple nucleotide changes. This instability of the virus genome in simian derived cell lines has made development of efficacious PRRSV vaccines difficult.

PAMs are the only known primary cells that have a high sensitivity to PRRSV infection and can produce relatively high yields of PRRSV. PAMs are the natural host for PRRSV and PRRSV propagated on PAMs may be more stable than PRRSV propagated on Vero derived cell lines. However, there are several disadvantages to using PAMs, such as: 1) difficulty in obtaining PAMs of consistent high quality, 2) difficulty of harvesting PAMs by pulmonary lavage and the risk of contamination by other organisms present in the lung, and 3) the high cost associated with maintaining a sufficient number of swine to provide the quantities of PAMs needed for large scale vaccine production or diagnostics. In addition, pulmonary lavage requires anesthetizing of swine thereby incurring further expense since a veterinarian is required to monitor the procedure and animal use permits are required. While PAMs are the natural host for PRRSV and produces high quantities of virus, the aforementioned disadvantages to using PAMs to grow PRRSV make PAMs unpractical for large scale production of live vaccines or diagnostics.

Even though PAMs are the natural host for PRRSV, the high degree of evolutionary divergence of PRRSV in the field indicates that propagation of vaccine strains of PRRSV on PAMs will not completely abrogate the high rate of divergence. The high rate of divergence of PRRSV in the field further suggests that the high rate of divergence observed for PRRSV grown on simian derived cell lines is not simply a function of propagating PRRSV on simian derived cell lines, but rather a function of the virus itself.

Live and killed virus vaccines to control PRRS have been available for several years, however these vaccines have been effective in less than 50% of herds. This fact is significant in that most herds are vaccinated 3 to 5 times a year. Furthermore, live virus vaccines cannot be used in pregnant sows as vaccine induced viremia may be transferred to offspring, resulting in disease symptoms.

Live virus vaccines are prepared from PRRSV isolated from infected simian cell lines (United States Patents 5,476,778 and 5,510,258). While passage of PRRSV on simian cell lines can result in attenuated virus suitable for use as a vaccine, the inherent instability of the virus genome when propagated on that cell lines makes it difficult to maintain virus at the appropriate level of attenuation. In some cases a more virulent strain of PRRSV may be produced by a higher passage level than at a lower passage level. The inherent instability of PRRSV appears to render attenuated live PRRSV vaccines ineffective in a short period of time, as witnessed by increasing incidences of PRRS outbreaks in vaccinated herds. Instability of attenuated live PRRSV vaccines requires the continual generation of new attenuated PRRSV vaccines. This is both time consuming and costly. Furthermore, the instability of the PRRSV genome makes it unlikely that a vaccine virus can be produced that contained markers that would clearly distinguish the vaccine virus from naturally occurring virus. This has prevented development of serological and PCR based diagnostics that

distinguish swine that have been vaccinated against PRRS from swine that have been infected with a naturally occurring PRRSV.

Killed vaccines while safer than live vaccines are even less effective than current live vaccines. Killed virus vaccines have been prepared from PRRSV isolated from PAMs and simian derived cell lines infected with PRRSV. While the PRRSV genome appears to be more stable when propagated on PAMs than on simian derived cell lines, both the process of PAM production and its associated high costs precludes the use of this procedure for large scale production of PRRSV vaccines. Generally, killed virus vaccines occupy a niche market whereby PRRSV from a particular farm is isolated and used to produce a vaccine specific for the PRRSV isolate currently found on the farm. Preparation of killed virus vaccines are also subject to the same degree of divergence during propagation as live vaccines.

New variants of PRRSV have appeared which overcome any protection provided by current vaccines. The dwindling efficacy of PRRSV vaccines is most likely a combination of naturally occurring PRRSV isolates evolving away from the protection afforded by the vaccine virus and the evolution of PRRSV vaccines towards over-attenuation. Clearly, a PRRSV vaccine that can be propagated for a longer period of time with limited divergence from the starting vaccine virus will maintain its protective efficacy for a longer period of time, thereby reducing the high level of vaccine failure now seen.

There is a clear need for PRRSV vaccines and diagnostics that are inexpensive, produce high titers of virus, and most importantly with reduced divergence of the virus genome compared to current PRRSV vaccines. Such a vaccine would ensure that changes within the viral genome during propagation of PRRSV for vaccine production would be more limited than is currently the case, therefore extending the useful life of PRRSV vaccine. Furthermore, such a stabilized PRRSV vaccine would enable production of vaccines that contained genetic markers that would enable the virus of the vaccine to be differentiated from

field strains of PRRSV. A marker inserted into a PRRSV having a genome not so stabilized would undergo cumulative mutations during propagation that would eventually render the marker unrecognizable by either serological and/or PCR based diagnostics.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation illustrating the genomic organization of PRRSV and EAV.

Figure 2 is a diagram illustrating plasmid p3ASP6.

Figure 3A is a diagram illustrating plasmid p3AT7.

Figure 3B is a diagram illustrating plasmidp4B.

Figure 4 is a diagram illustrating the *in vitro* ligation product 1SP6 comprising a 5,489 bp NdeI/XbaI DNA fragment from p3ASP6 containing the SP6 promoter operationally linked to EAV ORF 1a and the 7,769 bp EcoRI/XbaI DNA fragment from p4B containing EAV ORF 1b operationally linked to PRRSV ORFs 2-7.

Figure 5 is a diagram illustrating the *in vitro* ligation product 1T7 comprising a 5,489 bp NdeI/XbaI DNA fragment from p3AT7 containing the T7 promoter operationally linked to EAV ORF 1a and the 7,769 bp EcoRI/XbaI DNA fragment from p4B containing EAV ORF 1b operationally linked to PRRSV ORFs 2-7.

Figure 6 is a photograph of an agarose gel stained with ethidium bromide showing the PCR product of the recombinant EAV/PRRSV of the present invention produced by transfecting MRC-145 or MARC-145 cells with RNA transcribed from 1T7. Virus was isolated from infected cells and analyzed by RT-PCR. An internal control consisting of a synthetic RNA containing a 100 bp deletion of the 3' end of PRRSV was added to each PCR reaction to monitor the RT-PCR reaction. The RT-PCR products were resolved on an agarose gel and stained with ethidium bromide. Lane one is a DNA size marker. Lane 2 are the RT-

PCR reaction product of virus isolated from EAV infected cells showing the 289 bp internal control but not the 389 bp PRRSV . Lane 3 are the RT PCR reaction products of virus isolated from cells infected with the recombinant EAV/PRRSV chimera of the present invention showing the expected 389 bp PRRSV product contained within the present invention and the 289 bp internal control

Figures 7A-C are photomicrographs of cells infected with the recombinant EAV/PRRSV of the present invention produced by transfecting MRC-145 or MARC-145 cells with RNA transcribed from 1T7. The infected cells were reacted with a monoclonal antibody SR30 specific for PRRSV and then reacted with an alkaline-phosphatase conjugated anti-mouse antibody. The alkaline phosphatase was detected using the colormetric indicator BCIP and NBT. Fig. 7A is uninfected cells, Fig. 7B is cells infected with the present invention, and Fig. 7C is cells infected with PRRSV.

SUMMARY OF THE INVENTION

The present invention relates to nucleic acids and a method for producing such nucleic acids in which a sequence of nucleotides from an RNA virus is joined to a sequence of nucleotides from a different RNA virus to produce a recombinant genome capable of producing virus with decreased mutation rates.

The present invention relates to nucleic acids comprising a sequence of nucleotides encoding a polymerase from an RNA virus and ORFs 2-7 of PRRSV provided the sequence of nucleotides encoding the polymerase from the RNA virus is not a wild type PRRSV polymerase.

The present invention also relates to a nucleic acids which are comprised of a high fidelity RNA polymerase gene and the ORFs 2-7 from PRRSV.

The present invention further relates to the use of the above referenced nucleic acids to produce a recombinant virus to be used in a vaccine to protect swine from infection by PRRSV.

The present invention also relates to a method of producing the recombinant nucleic acids as well a method of producing virus containing these recombinant nucleic acids. Furthermore, the preferred embodiment of the present invention provides for use of said recombinant virus as a vaccine to protect swine from infection by PRRSV.

The present invention also relates to a method of detecting the recombinant nucleic acids in a population of swine that has been vaccinated with a vaccine containing such recombinant nucleic acids.

The foregoing merely summarizes certain aspects of the present invention and is not intended, nor should be construed, as limiting the invention in any manner. All patents and publications cited herein establish the state of the art and are hereby incorporated by reference in their entirety.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to nucleic acids and a method for producing such nucleic acids in which a sequence of nucleotides from an RNA virus is joined to a sequence of nucleotides from a different RNA virus to produce a recombinant viral genome capable of producing virus with decreased mutation rates.

An essential component of the life cycle of RNA viruses is replication of the viral genome with its own encoded RNA polymerase. RNA replicases and RNA-dependent polymerases, both hereinafter referred to as RNA virus polymerases, vary in their abilities to faithfully transcribe or replicate RNA templates. Many but not all RNA polymerases appear to lack a proof-reading function which is important for removing misincorporated nucleotides during replication. Therefore, RNA virus replication is generally thought to be error-prone

because base misincorporations are proof-read very inefficiently or not at all. For example, vesicular stomatitis virus polymerase error rate or mutation rate has been estimated to be approximately 10^{-3} per nucleotide (Holland *et al.*, Science (1982) vol. 215: 1577; Steinhauer and Holland (1986) J Virol 59:545). Other RNA viruses such as tobacco mosaic tobamovirus , have RNA polymerase mutation rates of less than 10^{-4} (Kearney *et al.* (1993) Virol 192:11-17). For other viruses such as polio virus, the error frequency ranged from 7×10^{-4} to 5.4×10^{-3} and was dependent on the reaction conditions (Ward *et al.*, (1988) J Virol 62:558-562). Other RNA virus RNA polymerase do possess proof-reading ability. Influenza is an example of an RNA virus whose replicase does have a proof-reading function (Isihama *et al.* (1986) J Biol Chem 261:10417). There is some evidence that particular mutations introduced into the RNA polymerase gene of a virus can affect the fidelity of the polymerases activity. For example, a single mutation in the polymerase gene of vesicular stomatitis virus resulted in an enhanced error rate (Pringle *et al.*, (1981) 39:377).

Variability in polymerase fidelity can be found for viral and non-viral RNA, DNA and RNA-dependent DNA polymerases. For example, human immunodificiency virus-1 (HIV-1) polymerase has an error rate of 1.4×10^{-4} on an RNA template and 1.7×10^{-4} on a DNA template whereas Moloney leukemia virus polymerase and polymerase 1 on RNA templates had error rates of less than 3.6×10^{-5} and 2.7×10^{-5}, repectively (Ji and Loeb, (1992) Biochem 31:954). Pandey *et al* (1996) Biochem 35:2168) reported that one mutation in the HIV-1 polymerase gene increased polymerase fidelity whereas another mutation decreased fidelity. Human telomerase is an example of a non-viral RNA-dependent polymerase that has an average error rate of 2×10^{-3} (Kreiter *et al.* (1995) Nuc Acids Symp. Ser 33:137).

It is clear that RNA polymerases from different organisms and viruses display considerable variability in fidelity. Some recent data indicates that for certain RNA polymerases, specific mutations in the gene encoding the polymerase can result in a

polymerase with either increased fidelity or decreased fidelity. However, for most RNA polymerases it is unclear how or which mutations introduced into a RNA polymerase with low fidelity would result in a RNA polymerase with greater fidelity.

Equine Ateritits Virus (EAV), an *Arterivirus* related to PRRSV, was first reported in 1958. (Fields Virology, Third Edition (1996), Fields, *et al.* (ed.), Lippincott-Raven Publishers, Philadelphia, PA. pp 1105-1120). EAV is responsible for a mild, often unrecognized respiratory disease in horses that resembles influenza. However, infection of pregnant mares can result in abortions and infection of young fouls produces more severe clinical signs. Similar to PRRSV, initial replication of EAV occurs in macrophages of the lung and then disseminates throughout the body. While EAV variants have been identified that vary in pathogenicity, to date only one serotype of EAV has been identified. Unlike PRRSV, EAV can be cultured *in vitro* on most cell lines that are derived from kidney cells from a variety of species.

The EAV RNA genome is very similar in structure to the PRRSV genome. The EAV genome is approximately 12.7 kb in length, polyadenylated and is organized into 8 open reading frames (ORFs). The first 189 nucleotides at the 5' end of the genome, 81 nucleotides between ORFs 1b and 2, and the last 392 nucleotides at the 3' end just upstream of the poly(A) are untranslated. The genome of EAV, like PRRSV, is transcribed into one genome length minus strand transcript that serves as a template for a genome length mRNA, encoding ORFs 1a and 1b, and a 3'-co-terminal nested set of six sub-genomic mRNAs. Like PRRSV, each mRNA is polyadenylated and the sub-genomic mRNAs each possess a leader sequence derived from the 5'-end of the viral genome.

The 5' end of the EAV genome spans approximately 9.8 kb, slightly smaller than the PRRSV genome, and encodes two large proteins, 1a and 1b similar to 1a and 1b of PRRSV. The ORF 1a protein is 1,728 amino acids and ORF 1b protein is 1,448 amino acids.

Expression of ORF 1b, like 1 b of PRRSV, is via a frameshift mechanism at a site that overlaps ORF 1a and ORF 1b.

Similar to PRRSV, EAV ORFs 2 through 7 overlap to varying degrees with their neighbors and the ORFs are read in alternate frames. ORFs 2, 5, and 7 are read in one frame, ORF 4 is read in another frame, and ORF 3 and 6 are read in the third frame and ORF 1b does not overlap with ORF 2. The encoded proteins for ORFs 2 through 7 are similar in size and function to the corresponding ORFs of PRRSV. ORF 2 encodes a 228 amino acid minor envelope glycoprotein, ORF 6 encodes a 163 amino acid nonglycosolated envelope protein, and ORF 5 encodes the 256 amino acid primary envelope glycoprotein which may have functions similar to ORF 5 in PRRSV. ORF 7 encodes a 111 amino acid nucleocapsid protein. ORFs 3 and 4 encode respectively 164 and 153 amino acid proteins of unknown function.

While the error rate for the EAV RNA polymerase has not be assayed, the lower rate of EAV genome divergence compared to PRRSV genome divergence suggests that the EAV RNA polymerase has increased fidelity.

Substitution of the polymerase gene of a first virus with a high fidelity polymerase gene from a second virus may be used as a general technique for obtaining a nucleic acid from which recombinant virus that are less mutagenic can be produced. In addition to the polymerase encoding sequence other portions of the genomic sequence of other RNA viruses may be included in a nucleic acid. The term "high fidelity polymerase" is meant as a relative term in which the polymerase that substitutes for the wild type polymerase has higher fidelity than the wild type polymerase. Examples of viruses that may be used in applying the aforementioned method may be viruses of the *Coronavirus*, *Togavirus*, and *Orthomyxovirus* families. For each virus family, the polymerase gene from one virus may be linked to the structural genes of another virus to produce a nucleic acid which when transfected into a host

cell produces a recombinant less mutagenic virus. This virus can then be used as a vaccine to provide protection against disease caused by the virus that contributed the structural genes.

The advantages of a recombinant virus with a reduced mutation rate several-fold. First, vaccines with reduced risk of loss or reduction of efficacy can be produced. Second, the high fidelity RNA polymerase gene can be used as a marker that allows organisms vaccinated with such a vaccine to be distinguished from organisms naturally infected with wild type strains of virus or other vaccines.

In its broadest aspect, the invention comprises compositions and methods for making and using the compositions, wherein the composition comprises a nucleic acid which itself comprises a virus in which the native RNA polymerase of the virus is replaced with an RNA polymerase having higher fidelity than the native RNA polymerase of the virus.

A preferred embodiment of the present invention provides for a nucleic acid comprising a high fidelity RNA polymerase gene linked to PRRSV ORFs 2-7. The high fidelity RNA polymerase gene replaces the PRRSV RNA polymerase and therefore provides a nucleic acid that encodes a polymerase that will produce a recombinant PRRSV which has reduced divergence of its viral genome during replication. A more preferred embodiment of the present invention provides a recombinant PRRSV comprising genetic elements from two Arteriviruses, specifically ORFs 2-7 from PRRSV and ORFs 1a and 1b of EAV. There are fewer reported strains of EAV than PRRSV, the difference in mutation rates between EAV and PRRSV is a result of enhanced fidelity of the EAV RNA polymerase compared to the fidelity of the PRRSV RNA polymerase. Therefore, the EAV RNA polymerase gene comprising the preferred embodiment of the present invention allows propagation of the recombinant virus with a reduced mutation rate.

The present invention provides for nucleic acids which may have additional coding or non-coding nucleotide sequences interspersed or added to the 5' or 3' ends of the

polymerase/structural gene encoding nucleic acid. For example, a nucleic acid of the present invention comprises the 5' untranslated leader sequence and open reading frames (ORFs) 1a and 1b of EAV joined to the ORFs 2 through 7 and 3' untranslated sequences of PRRSV. In a second example, the recombinant PRRSV comprises the 5' untranslated leader sequences and ORFs 1a and 1b of EAV joined to the 5' end of the PRRSV sequence encoding ORFs 2 through 7, and the 3' untranslated sequences of EAV joined to the 3' end of the PRRSV sequence encoding ORFs 2 through 7. In a third example, the recombinant PRRSV comprises the 5' untranslated leader sequence of PRRSV joined to the 5' end of the EAV sequence encoding ORFs 1a and 1b and PRRSV sequence encoding ORFS 2 through 7 joined to the 3' end of the EAV sequence.

The present invention also relates to a method of producing recombinant virus from the recombinant nucleic acid sequence. For example the nucleic acid sequence may be either RNA or DNA which when transfected into a host cell produces recombinant virus. A preferred embodiment of the present invention is a recombinant PRRSV which comprises a nucleic acid having the ORFs 1a and 1b from EAV and the ORF 2-7 from PRRSV. In one embodiment of the method for producing recombinant PRRSV the EAV and PRRSV sequences are made into DNA and the DNA fragments are cloned into a single DNA plasmid downstream from an RNA promoter in the order, 5' leader sequence, EAV ORF 1a and 1b, PRRSV ORFs 2 through 7, and 3' untranslated sequences to produce a recombinant DNA clone. It is understood that any of the aforementioned embodiments of the example of the present invention may be cloned in the described manner. The plasmid containing the recombinant DNA clone is transfected into cells. Within the cell, the recombinant DNA is transcribed into a positive strand recombinant RNA genome comprising the EAV and PRRSV sequences. The recombinant RNA genome replicates in the cell and is properly packaged in the cell as recombinant infectious PRRSV of the present invention. The

recombinant infectious PRRSV is recovered from the cell and is used to infect cells to make large quantities of recombinant PRRSV for use as a vaccine against PRRSV.

In another embodiment of the method for producing the recombinant PRRSV of the present invention, the aforementioned DNA plasmid comprising the EAV and PRRSV sequences is transcribed *in vitro* into a positive strand recombinant RNA genome. The recombinant RNA genome is transfected into cells wherein it undergoes replication and is packaged into infectious virus. The infectious recombinant PRRSV is recovered from the transfected cells and is used to infect cells to make large quantities of recombinant PRRSV for use as a vaccine against PRRSV.

The present invention also relates to the production of a vaccine which employs the recombinant virus of the present invention. A preferred embodiment of the vaccine of the present invention comprises a recombinant PRRSV which comprises a nucleic acid having the ORFs 1a and 1b from EAV and the ORF 2-7 from PRRSV

Recombinant PRRSV of the present invention is used to produce live vaccines to protect swine against PRRS. Virus of the present invention is concentrated, frozen, and stored at -70° C. or freeze-dried and stored at 4 °C. Virus of the present invention for vaccination is mixed with a physiological carrier, such as saline solution and/or adjuvant, such as aluminum hydroxide, approved for use in livestock to the appropriate dosage and administered either by oral or nasal exposure or by injection.

The recombinant PRRSV of the present invention can be used to make an inactivated PRRSV vaccine by methods well-known to the art that includes treatment with formalin or binary ethyleneimine (BEI). The inactivated recombinant PRRSV is mixed with a physiological carrier, such as saline solution and/or adjuvant, such as aluminum hydroxide, approved for use in livestock to the appropriate dosage and administered either by oral or nasal exposure or by injection.

In another embodiment of the present invention a marker sequence is inserted into the recombinant PRRSV so that the virus may be uniquely identified by the marker sequence. The marker is a nucleic acid sequence that is not found in PRRSV strains. The marker will therefore be useful in the identification of the recombinant PRRSV such that one may distinguish between cells infected with the recombinant PRRSV and those infected with some wild-type PRRSV or cells not infected by PRRSV-like virus. In a preferred embodiment of the present invention the marker sequence is derived from ORF 1a and 1b of EAV. In this embodiment, ORF 1a and 1b serve the dual role of encoding a polymerase for the recombinant PRRSV and as a marker for the unique identification of this recombinant virus. Identification of the recombinant PRRSV for using such a marker can be accomplished by techniques well-known in the art such as the polymerase chain reaction (PCR), hybridization techniques, and reverse transcriptase PCR (RT-PCR) described in Molecular Cloning: A Laboratory Manual, Second Edition (Sambrook, Fritsch, Maniatis, eds. (1989), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

The invention will be illustrated in further detail in the following by means of examples, which are intended merely to illustrate the invention, not to limit it in any way. Those skilled in the art will appreciate that modifications and variations of the following can be made without deviating from the spirit or scope of the invention.

EXAMPLES

Example 1

Isolation and Cloning of ORFs 1a and 1b from EAV

EAV 5' untranslated end, ORF 1a, and ORF 1b were cloned from EAV RNA by purifying EAV RNA, making cDNA to the RNA, PCR amplyfing the cDNA by polymerase chain reaction (PCR), and cloning the PCR amplified DNA fragments as follows.

The Bucyrus strain of EAV (ATCC accession number VR-796) was propagated on MRC-145 cells (monkey kidney line available from the National Veterinary Services Laboratories, P.O. Box 844, Ames, IA 50010 of the United States Department of Agriculture, Animal and Plant Health Inspection Service, Biotechnology, Biologics, and Environmental Protection) to make a stock of infectious virus. The RNA from the infectious virus was purified using TriZol Reagent (GIBCO-BRL, Gaithersburg, MD) as follows. EAV infected cells on 35 mM tissue culture dishes were lysed by adding 1 ml of Trizol Reagent to each dish and pipetting the cell lysates several times. The cell lysates were transferred to microcentrifuge tubes (two microcentrifuge tubes per 35 mm dish) and incubated at room temperature for 5 minutes. Then 0.2 ml of chloroform per ml of Trizol Reagent was added and the tubes vigorously shaken for 15 seconds and then incubated at room temperature for 3 minutes. The tubes were centrifuged at 12,000 x g for 15 minutes at 4°C. Afterwards, the aqueous phase containing the RNA was transferred to a new tube and the RNA was precipitated by addition of 0.5 ml isopropanol per ml of Trizol Reagent. The samples were incubated for 10 minutes at room temperature, then centrifuged for 15 minutes at 12,000 x g at 4°C. The supernatant fractions were removed and the RNA pellets were washed once with 1 ml of 75% (v/v) ethanol. The pellets were air dried for 5 minutes before dissolving in 50 µl of RNAse-free water.

The purified EAV RNA was used as a template to make cDNA using Superscript II reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) according to the following procedure. 0.5 to 1.0 µg EAV RNA was added to 1 ul 500 µg/µl oligo $d(T)_{12-18}$ and brought up to a final volume of 12 µl. The solution was incubated at 70°C for 10 minutes to denature the RNA, then chilled on ice for 10 minutes. Then 4 µl of 5x Superscript II buffer containing 250 mM Tris-HCl pH 8.3, 375 mM Kcl, and 15 mM MgCl2 (BRL-GIBCO), 2 µl 0.1 M DTT (dithiothreitol), and 1 µl of 10 mM deoxynucleotide solution containing 10 mM each of the

four deoxynucleotides (dNTP), guanosine triphosphate (dGTP), adenosine triphosphate (dATP), cytosine triphosphate (dCTP), and thymidine triphosphate (dTTP) was added to the denatured RNA solution. The final concentration of the reaction mixture was 50 ng/μl RNA, 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTP. Finally, 1 μl of Superscript II containing 1 unit of enzyme was added and the reaction was incubated 50 minutes at 42°C. Afterwards, the reaction was heated at 70°C for 15 minutes, then stored frozen until needed.

ORFs 1a and 1b were PCR amplified from EAV cDNA as three separate DNA fragments (82A, 887, and 774) and seperately cloned. The cloned DNA fragments were then assembled into a clone containing the entire EAV ORF 1a and ORF 1b. To facilitate construction of the complete ORF 1a and ORF 1b clone, each PCR primer set contained primers with specific restriction enzyme sites near the 5' ends of the primers to allow the cloned PCR DNA fragments to be released by digestion with the appropriate combination of restriction enzymes and the released fragments cloned in the appropriate order.

DNA primer set EAV-1 (5'-GTTGCTAGCTC GAAGTGTGTA TGGTGCCATA TACGGCTCAC CACCATATAC ACTGC) with the *Nhe*I restriction enzyme site underlined and EAV-2 (5'-CATGGTCGAC AACGGTCACA CCG) with the *Sal*I restriction enzyme site underlined, SEQ ID NO: 1 and SEQ ID NO: 2, respectively were used to PCR amplify nucleotides 2 through 2599 of the EAV genome to produce DNA fragment 82A, 2.6 kb in length. The PCR reaction volume was 100 μl and consisted of 5 μl of the abovementioned cDNA reaction mixture, 1 μM each primer, 0.2 mM dNTP, 20 mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% (v/v) Triton X-100, 100 μg/ml (w/v) bovine serum albumen, and 1 unit Taq Plus DNA polymerase (Stratagene, La Jolla, CA). The PCR reaction was performed in a GeneAmp 2400 thermal cycler (Perkin Elmer Cetus, Norwalk, CT) under the following conditions: 94°C for 4 minutes, then 40 cycles consisting of 93°C for

45 seconds, 50°C for 30 seconds, and 72°C for 4 minutes, followed by a 15 minute incubation at 72°C.

DNA primer set EAV-3 (5'-CGTTGTCGAC CATGCTCTTT ACAACC) with the *Sal*I restriction enzyme site underlined and EAV-4 (5'-GTCTCTAGAG TCAGCAAAGG TCC) with the *Xba*I restriction enzyme site underlined, SEQ ID NO: 3 and SEQ ID NO: 4, respectively were used to PCR amplify nucleotides 2586 through 5450 of the EAV genome to produce DNA fragment 887, 2.9 kb in length. The PCR reaction volume was 100 µl and consisted of 5 µl of the abovementioned cDNA reaction mixture, 1 µM each primer, 0.2 mM dNTP, 20 mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% (v/v) Triton X-100, 100 µg/ml (w/v) bovine serum albumen, and 1 unit Taq Plus DNA polymerase (Stratagene, La Jolla, CA). The PCR reaction was performed in a GeneAmp 2400 thermal cycler (Perkin Elmer-Cetus, Norwalk, CT) under the following conditions: 94°C for 4 minutes, then 40 cycles consisting of 93°C for 45 seconds, 60°C for 30 seconds, and 72°C for 4 minutes, followed by a 15 minute incubation at 72°C.

DNA primer set EAV-5 (5'-GACTCTAGAG ACAAGGGTTT CG) with the *Xba*I restriction enzyme site underlined and EAV-6 (5'-AAAGATGAAG TGGGATCCTC CCACGG) with the *Bam*HI restriction enzyme site underlined, SEQ ID NO: 5 and SEQ ID NO: 6, respectively were used to PCR amplify nucleotides 5439 through 9149 of the EAV genome to produce DNA fragment 774, 3.7 kb in length. The PCR reaction volume was 100 µl and consisted of 5 µl of the abovementioned cDNA reaction mixture, 1 µM each primer, 0.2 mM dNTP, 20 mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% (v/v) Triton X-100, 100 µg/ml (w/v) bovine serum albumen, and 1 unit Taq Plus DNA polymerase (Stratagene, La Jolla, CA). The PCR reaction was performed in a GeneAmp 2400 thermal cycler (Perkin Elmer-Cetus, Norwalk, CT) under the following conditions: 94°C

for 4 minutes, then 40 cycles consisting of 93°C for 45 seconds, 50°C for 30 seconds, and 72°C for 4 minutes, followed by a 15 minute incubation at 72°C.

DNA primer set EAV-7 (5'-GGAGGATCCC ACTTCATCTT TTCC) with the *Bam*HI site underlined and EAV-8 (5'-TGCCTCGAGC ACAGAAATAG CAAAATCAGC) with the *Xho*I site underlined, SEQ ID NO: 7 and SEQ ID NO: 8, respectively were used to PCR amplify nucleotides 9129 through 9811 of the EAV genome to produce DNA fragment 78A, 682 bp in length. The PCR reaction volume was 100 µl and consisted of 5 µl of the abovementioned cDNA reaction mixture, 1 µM each primer, 0.2 mM dNTP, 20 mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% (v/v) Triton X-100, 100 µg/ml (w/v) bovine serum albumen, and 1 unit Taq Plus DNA polymerase (Stratagene, La Jolla, CA). The PCR reaction was performed in a GeneAmp 2400 thermal cycler (Perkin Elmer-Cetus, Norwalk, CT) under the following conditions: 94°C for 4 minutes, then 40 cycles consisting of 93°C for 45 seconds, 50°C for 30 seconds, and 72°C for 1 minute, followed by a 15 minute incubation at 72°C.

Construction of the infectious clone of the present invention with the above mentioned PCR DNA fragments is described in Example 3 below.

Example 2

Isolation and Cloning of ORFs 2-7 from PRRSV

PRRSV ORF 2 through 7 and the 3' untranslated end were made from PRRSV RNA by purifying PRRSV RNA, making cDNA to the RNA, PCR amplyfing the cDNA by polymerase chain reaction (PCR), and cloning the PCR amplified DNA fragment as follows.

The PRRSV strain PUNG was propagated on MRC-145 cells to make a stock of infectious virus. The RNA from the infectious virus was purified using TriZol Reagent (GIBCO-BRL, Gaithersburg, MD). PRRSV infected cells on 35 mM tissue culture dishes were lysed by adding 1 ml of Trizol Reagent to each dish and pipetting the cell lysates several

times. The cell lysates were transferred to microcentrifuge tubes (two microcentrifuge tubes per 35 mm dish) and incubated at room temperature for 5 minutes. Then 0.2 ml of chloroform per ml of Trizol Reagent was added and the tubes vigorously shaken for 15 seconds and then incubated at room temperature for 3 minutes. The tubes were centrifuged at 12,000 x g for 15 minutes at 4°C. Afterwards, the aqueous phase containing the RNA was removed to a new tube and the RNA was precipitated by addition of 0.5 ml isopropanol per ml of Trizol Reagent. The samples were incubated for 10 minutes at room temperature, then centrifuged for 15 minutes at 12,000 x g at 4°C. The supernatant fractions were removed and the RNA pellets were washed once with 1 ml of 75% (v/v) ethanol. The pellets were air dried for 5 minutes before dissolving in 50 µl of RNAse-free water.

DNA primer set PRRSV-1 (5'-GGCCTCGAGT GAAATGAAAT GGGGTCCATGC AAAGCC) with the *Xho*I restriction enzyme site underlined and PRRSV-2 (5'-GCGAATTC-$(T)_{52}$-AATTTCGGCCGCATGGTTCTCGCCAATTAAATCTCACC) with the *Eco*RI restriction enzyme site underlined, SEQ ID NO: 9 and SEQ ID NO: 10, respectively were used to PCR amplify nucleotides 72 through 3423 of the PRRSV genome to produce DNA fragment 87, 3.4 kb in length. The sequence numbering corresponds to the numbering system published by Murtaugh *et al.* (Archives of Virology, 1995, vol 140 pages 1451-1460) for the BIAH-001 strain of PRRSV. The PCR reaction volume was 100 µl and consisted of 5 µl of the abovementioned cDNA reaction mixture, 1 µM each primer, 0.2 mM dNTP, 20 mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% (v/v) Triton X-100, 100 µg/ml (w/v) bovine serum albumen, and 1 unit Taq Plus DNA polymerase (Stratagene, La Jolla, CA). The PCR reaction was performed in a GeneAmp 2400 thermal cycler (Perkin Elmer-Cetus, Norwalk, CT) under the following conditions: 94°C for 4 minutes, then 40 cycles consisting of 93°C for 45 seconds, 60°C for 30 seconds, and 72°C for 4 minutes, followed by a 15 minute incubation at 72°C.

Construction of the infectious clone of the present invention with the abovementioned PCR DNA fragments is described in Example 3 below.

Example 3

Construction of Infectious Recombinant PRRSV

This example describes the methods for constructing the infectious clone of the present invention. The methods used molecular biology techniques well known to those in the art and are documented in Molecular Cloning: A Laboratory Manual, Second Edition (Sambrook, Fritsch, Maniatis, eds. (1989), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

To enable infectious EAV-PRRSV positive strand RNA to be made *in vitro,* a second embodiment of the present invention, an RNA polymerase promoter operationally linked to the 5' end of EAV ORF 1a Examples of RNA polymerase promoters are bacteriophage promoters T7 or Sp6.

To operationally link any of the above mentioned RNA polymerase promoters to the 5' end of EAV ORF 1a, a DNA primer set consisting of 5'-GCCCATATGA TTTAGGTGAC ACTATAGCTC GAAGTGTGTA TGGTGCCATA TACGGCTCAC CACCATATAC ACTGC (SEQ ID NO.: 21) with the *Nde*I restriction enzyme site and the SP6 promoter and EAV-2 (SEQ ID NO.:2) was used to PCR amplify nucleotides 2 through 2599 of the EAV genome to produce 2.6 kb DNA fragment SP682A with EAV ORF 1a operationally linked to the SP6 promoter or DNA primer set consisting of 5-GCCCATATGT AATACCAGTC ACTATAGCTC GAAGTGTGTA TGGTGCCATA TACGGCTCAC CACCATATAC ACTGC (SEQ ID NO.:22) with the *Nde*I restriction enzyme site and the T7 promoter and EAV-2 (SEQ ID NO.:2) was used to PCR amplify nucleotides 2 through 2599 of the EAV genome to produce 2.6 kb DNA fragment T782A with EAV ORF 1a operationally linked to the T7 promoter. The PCR reaction volume was 100 µl and consisted of 100 µg of DNA

fragment 82A, 1 μM each primer, 0.02 mM dNTP, 20 mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% (v/v) Triton X-100, 100 μg/ml (w/v) bovine serum albumen, and 1 unit TAQ PLUS DNA polymerase. The PCR reaction was performed in a GeneAmp 2400 model thermal cycler under the following conditions: 94°C for 4 minutes, then 40 cycles consisting of 93°C for 45 seconds, 50°C for 30 seconds, and 72°C for 4 minutes, followed by a 15 minute incubation at 72°C.

Plasmid p1A was digested with restriction enzymes *Sal*I and *Xba*I and PCR amplified DNA fragment 887 digested with *Sal*I and *Xba*I was cloned between the *Sal*I/*Xba*I sites of p1A to produce plasmid p2A. PCR amplified EAV DNA fragments SP682A and T782A were digested with restriction enzymes *Nde*I and *Sal*I. The 2.6 kb DNA fragments were cloned between restriction enzyme sites *Nde*I and *Sal*I of plasmid p2A that had been digested with *Nde*I and *Sal*I to produce plasmids p3ASP6 (Figure 2) and p3AT7 (Figure 3), respectively. Plasmid p1B was digested with restriction enzymes *Xba*I and *Bam*HI and PCR amplified EAV DNA fragment 774 digested with *Xba*I and *Bam*HI was cloned between the *Xba*I/*Bam*HI sites of p1B to produce plasmidp2B. Plasmid p2B was digested with *Bam*HI and *Xho*I and PCR amplified EAVDNA fragment 78A digested with *Bam*HI and *Xho*I was cloned betweent he *Bam*HI/*Xho*I sites of p2B to produce plasmid p3B. Plasmid p3B was digested with *Xho*I and *Eco*RI and PCR amplified PRRSV DNA fragment 87 digested with *Xho*I and *Eco*RI was cloned between the *Xho*I/*Eco*RI sites of p3B to produce plasmid p4B (Figure 4). Finally, plasmids p3ASP6 and p3AT7 were digested with restriction enzymes *Nde*I and *Xba*I to produce 5,489 bp DNA fragments, 3ASP6 and 3AT7 respectively. Similarly, plasmid p4B was digested with restriction enzymes *Xba*I and *Eco*RI and the 7769 bp DNA fragment containing EAV ORF 1b and PRRSV ORFs 2-7 was *in vitro* ligated to either DNA fragment 3ASP6 or 3AT7 to produce ligation products 1SP6 or 1T7 (Figure 5), respectively for *in vitro* transcription. DNA ligations were performed using T4 DNA ligase

(supplied by Invitrogen, Carlsbad, CA) as desribed in Sambrook *et al.* (Molecular Cloning: A Laboratory Manual, Second Edition, (Sambrook, Fritsch, Maniatis, eds. (1989), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

An alternative method to operationally linked the Sp6 or T7 promoters to EAV ORF 1a is as follows. Complementary DNA linker pairs 1 and 2 is replaced with complementary linker pairs 5 (5'-CTAGTAATAC GACTCACTAT AGGCTAGCTC TAGAGATATC GAATTCGGTA C, SEQ ID NO: 15) and 6 (5'-CGAATTCGAT ATCTCTAGAG CTAGCCTATAG TGAGTCGTAT TA, SEQ ID NO: 16) containing a T7 bacteriophage promoter (sequence underlined for Linker-5) upstream of the functional *Nhe*I site, or complementary linker pairs 7 (5'-CTAGATTTAG GTGACACTAT AGCTAGCTTA GAGATATCGA ATTCGGTAC, SEQ ID NO: 17) and 8 (5'-CGAATTCGATA TCTCTAGAGC TAGCCTATAG TGAGTCGTAT TA,SEQ ID NO: 18) containing an Sp6 bacteriophage promoter (sequence underlined for Linker-7) upstream of the functional *Nhe*I site. Linker pair 5 and 6 is inserted between the *Nhe*I and *Kpn*I sites of pBK-CMV to produce plasmid p1A-T7. The plasmid p1A-T7 is digested with restriction enzymes *Nhe*I and *Xba*I and used to clone *Nhe*I/*Xba*I digested EAV DNA fragment 82 which is then used instead of plasmid p2A to make the present invention operationally linked to a T7 promoter. Linker pair 7 and 8 is inserted between the *Nhe*I and *Kpn*I sites of pBK-CMV to produce plasmid p1A-Sp6. The plasmid p1A-Sp6 is digested with restriction enzymes *Nhe*I and *Xba*I and used to to clone *Nhe*I/*Xba*I digested EAV DNA fragment 82 which is then used instead of plasmid p2A used to make the present invention operationally linked to an Sp6 promoter. Since these linkers contain an *Nhe*I site upstream of the T7 promoter which is necessary for cloning EAV DNA fragment 82, the linkers were designed to render non-functional the *Nhe*I site in pBK-CMV which is downstream of the promoter contained within the linkers. Therefore, each plasmid is then used to produce species of the second embodiment of the

present invention with each species operationally linked to either the T7 or Sp6 RNA polymerase promoters. These promoters *in vitro* produce full length RNA transcripts of the present invention that are infectious and produce infectious virus when transfected into cells. The species of the present invention comprising the T7 promoter is pEP-T7 and the species comprising the Sp6 promoter is pEP-Sp6.

An alternative method to operationally link the Sp6 or T7 promoter to the recombinant virus of the present invention is to use primer EAV-9 containing nucleotides 5'-GTTGCTAGCA TTTAGGTGAC ACTATAGCTC GAAGTGTGTA TGGTGCCATA TACGGCTCAC CATATACACT GC (SEQ ID NO: 19) which incorporates the Sp6 promoter (underlined) just downstream of the *Nhe*I site instead of primer EAV-1 to produce by PCR the 2,597 bp DNA fragment containing EAV ORF 1a. The PCR amplified DNA fragment is then cloned between the *Nhe*I/*Sal*I sites of plasmid p1A instead of DNA fragment 82A to make plasmid p2A-Sp6. Plasmid p2A-Sp6 is used instead of p2A to make the infectious clone of the present invention as described in the aforementioned manner. Primer EAV-10 containing nucleotides 5'-GTTGCTAGCT AATACGACTC ACTATAGCTC GAAGTGTGTAT GGTGCCATAT ACGGCTCACC ACCATATACACT GC (SEQ ID NO: 20) which incorporates the T7 promoter (underlined) just downstream of the NheI site is used instead of primer EAV-1 to produce by PCR the 2,597 bp DNA fragment containing EAV ORF 1a. The PCR DNA fragment is then cloned between the *Nhe*I/*Sal*I sites of plasmid p1A instead of DNA fragment 28A to make plasmid p2A-T7. Plasmid p2A-T7 is used instead of p2A to make the infectious clone of the present invention as described in the aforementioned manner.

To construct another embodiment of the infectious EAV-PRRS recombinant virus of the present invention linked to the CMV promoter, plasmid pBK-CMV (sold by Stratagene, La Jolla, CA) is modified as follows. To clone the PCR amplified EAV ORF 1a, pBK-CMV

is digested with restriction enzymes *Xba*I and *Kpn*I and complementary DNA linker pairs containing restriction enzymes sites *Xba*I, *Eco*RV, EcoRI and *Kpn*I (Linker-1, 5'-CTAGAGATATC GAATTCGGTA C and Linker-2, 5'-CGAATTCGAT ATCT; SEQ ID NO: 11 and SEQ ID NO: 12, respectively), is inserted between the *Xba*I/*Kpn*I sites to produce plasmid p1A. To clone PCR amplified EAV ORF 1b and PRRSV ORFs 2-7, pCMV-BK is digested with *Sac*I and *Kpn*I and complementary DNA linker pairs containing restriction enzyme sites *Sac*I, *Xba*I, *Pst*I, *Bam*HI, *Xho*I, *Eco*RI, and *Kpn*I (Linker-3, 5'-CTCTAGACTG CAGGGATCCC TCGAGGAATT CGGTAC and Linker-4, 5'-CTCTAGACTG CAGGGATCCC TCGAGGAATT CGGTAC; SEQ ID NO: 13 and SEQ ID NO 14) is inserted between the *Sac*I/*Kpn*I sites to produce plasmid p1B. For both of these plasmids any DNA fragment that is inserted next to the *Nhe*I site is operationally linked to a CMV promoter.

PCR amplified EAV DNA fragment 82A was digested with restriction enzymes *Nhe*I and *Sal*I. DNA fragment 82A contains an *Nhe*I site 500 bp from the 5' end of 82A, therefore the *Nhe*I/*Sal*I digest of 82A produces a 500 bp DNA fragment and a 2.1 kb DNA fragment. The 2.1 kb DNA fragment was cloned between restriction enzyme sites *Nhe*I and *Sal*I of plasmid p1A that has been digested with *Nhe*I and *Sal*I to produce plasmid p2A'. Then p2A' was digested with *Nhe*I and the 500 bp DNA fragment was cloned into the *Nhe*I site to produce plasmid p2A. Plasmid p2A was digested with restriction enzymes *Sal*I and *Xba*I and PCR amplified DNA fragment 887 digested with *Sal*I and *Xba*I was cloned between the *Sal*I/*Xba*I sites of p2A to produce plasmid p3A. Plasmid p1B was digested with restriction enzymes *Xba*I and *Bam*HII and PCR amplified EAV DNA fragment 774 digested with *Xba*I and *Bam*HI was cloned between the *Xba*I/*Bam*HI sites of p1B to produce plasmid p2B. Plasmid p2B was digested with *Bam*HI and *Xho*I and PCR amplified EAV DNA fragment 78A digested with *Bam*HI and *Xho*I was cloned between the *Bam*HI/*Xho*I sites of p2B to

produce plasmid p3B. Plasmid p3B was digested with *Xho*I and *Eco*RI and PCR amplified PRRSV DNA fragment 87 digested with *Xho*I and *Eco*RI was cloned between the *Xho*I/*Eco*RI sites of p3B to produce plasmid p4B (Figure 4). Finally, plasmid p4B was digested with restriction enzymes *Xba*I and *Eco*RI and the DNA fragment containing EAV ORF 1b and PRRSV ORFs 2 through 7 is inserted between the *Xba*I/*Eco*RI sites of p3A digested with restriction enzymes *Xba*I and *Eco*RI to produce one embodiment of the infectious clone of the present invention, pEP. This embodiment has the cytomegalovirus (CMV) immediate early promoter operationally linked to the 5' end of EAV ORF 1a. The EAV-PRRSV infectious clone pEP is transfected into cells and transcription using the CMV promoter produces an infectious positive strand RNA. While this example of the present invention is operationally linked to the CMV promoter, the invention can be operationally linked to other promoters such as the herpes simplex virus thymidine kinase promoter, SV40 promoter, or the rous sarcoma virus long terminal repeat promoter.

In the manner described in the above examples, infectious clones comprising all of the abovementioned embodiments and species of the present invention are constructed using ORFs 2 through 7 of PRRSV isolate BIAH-001 (ATCC accession number VR-2332).

Example 4

DNA Transfections

Plasmid pEP DNA is isolated from transformed *E.coli* and purified by cesium chloride gradient centrifugation. Cells susceptible to PRRSV replication will be transfected with one to five micorgrams of plasmid DNA using any of the transfection technologies such as calcium phosphate precipitation, DEAE-Dextran, or Polybrene as described in Molecular Cloning: A Laboratory Manual, Second Edition (Sambrook, Fritsch, Maniatis, eds. (1989), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) or Lipofectin (GIBCO-BRL,

Gaithersburg, MD) according to the manufacturer's directions. Viruses of the present invention are identified by cytopathic effect (CPE) and propagated by serial passage on suitable cells to produce sufficient virus to use as a live virus vaccine.

Example 5

RNA transfections

To a cell line susceptible to PRRSV replication with *in vitro* transcribed RNA, plasmids 1Sp6 and 1T7 were linearized with a restriction enzyme *Eco*RI which cleaved beyond the 3' terminus of the infectious clone of the present invention. Complementary RNA was transcribed *in vitro* using available *in vitro* transcription technologies exactly as described in Molecular Cloning: A Laboratory Manual, Second Edition, (Sambrook, Fritsch, Maniatis, eds. (1989), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). Cells were transfected with the *in vitro* transcribed RNA with LIPOFECTIN, DMRIE-C (DMRIE-C is a trademark of GIBCO-BRL, Gaithersburg, MD) or electroporation using a BioRad Gene Pulser electroporation apparatus (BIORAD and GENE PULSER are trademarks of BioRad Hercules, CA). Electroporation was done as follows. MRC-145 cells from a monolayer culture were trypsinized, washed once with phosphate buffered saline, and resuspended in DMEM medium (Dulbecco's Modified Eagle's Medium sold by GIBCO-BRL, Gaithersburg, MD) containing 10% calf serum to a final concentration of 3.75×10^6 cells/ml. 1 μg of viral RNA was added to 800 μl of cell suspension along with 1 unit of Rnasin in a sterile 0.4 cm electroporation cuvette and incubated 10 minutes at room temperature. Then two consecutive pulses of 300 volts (0.7 kv/cm) and 960 μFD were applied to the cells. After electroporation cells were allowed to recover for 10 minutes at room temperature before they were seeded on 60 mm tissue culture plates with DMEM media containing 10% calf serum and incubated at 37°C. Plaques typically appeared 3 days post-electroporation.

Transfections with LIPOFECTIN was done using MRC-145 cells according to the instructions provided by the manufacturerer (GIBCO-BRL, Gaithersburg, MD) with 1 to 5 μg of viral RNA. Plaques typically appeared 3 days after transfection. DMRIE-C transfections were done using MRC-145 cells according to the manufacturer's instructions (GIBCO-BRL, Gaithersburg, MD) using 5 μg of viral RNA. Plaques typically appeared 3 days post-transfection. Viruses of the present invention produced by transfection of RNA are identified by cytopathic effect (CPE) and purified. The purified viruses are propagated on suitable cells to provide sufficient virus for live virus vaccines.

Recombinants were identified using the reverse transcription polymerase chain reaction (RT-PCR). At four days post-transfection or post-electroporation, RNA was isolated from 200 ul of tissue culture supernatant using the QIAmp kit (sold by Qiagen, Chatsworth, CA) according to the manufacturer's specifications. One fifth of the isolated RNA was used to synthesize cDNA using SuperScript Reverse Transcriptase II (RNaseH-) and 500 ng of oligo d(T) (all sold by Gibco BRL, Gaithersburg, MD) by annealing oligo d(T) to the RNA template at 70°C for 10 minutes, following by reverse transcription using 200 units of reverse transcriptase at 42°C for 50 minutes and than at 72°C for 10 minutes. cDNA was then amplified using 2.5 Units of TaqPlus Long (sold by Stratagene, La Jolla, CA) in reaction volume comprising 20 mM Tris-HCl, pH 8.8, 10 mM KCL, 10 mM (HN4)2SO4, 2mM MgSO4,).1% (v/v) Triton-X-100, 100 ug/ml bovine serum albumen with 20 pmol of the primary primer pairs 5' -GTTTCAGCGG AACAATGG (SEQ ID NO: 23) and 5' CCCTAATTGA ATAGGTGAC (SEQ ID NO: 24) for 20 cycles after an initial denaturation step at 93°C for 4 minutes using the following parameters: 93°C for 45 seconds, 55°C for 30 sec and 72°C for 45 seconds. A final extension at 72°C for 9 minutes was used to enhance the amount of amplified product. The amplified product was then subjected to additional cycles of amplification using the "nested" primer pairs 5'-GTCTGGATTG ACGACAGACA (SEQ

ID NO: 25) and 5'-AACCACGCAT TTGTCGTCCG (SEQ ID NO.:26) using the abovementioned PCR conditions. The PCR reactions included an internal control which consisted of a synthetic RNA template having the 3' end of the PRRSV genome with a 100 bp deletion. PCR amplification using the above mentioned primers produces a 289 bp DNA product using the internal control template whereas PRRSV or the recombinant EAV/PRRSV chimera of the present invention produces a 389 bp DNA product. PCR amplified products were separated on 2.0% 1X TAE (0.04 M Tris-acetate and 0.002M EDTA) agarose gels and visualized by staining with 10 μg/ml of ethidium bromide as described in Molecular Cloning: A Laboratory Manual, Second Edition (Sambrook, Fritsch, Maniatis, eds (1989), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). Figure 6 shows the PCR reaction results indicating that the cells produced infectious virus of the present invention.

Recombinant were also identified using the immunoperoxidase monolayer assay (IPMA) as described in Antibodies: A laboratory Manual, (Harlow and Lane, eds (1988), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). MARC 145 cells were infected with lysates from cells previously transfected or electroporated with *in vitro* transcribed RNA. At four days post-transfection or post-electroporation, the infected cells were washed twice with 0.01M Phosphate buffered saline (PBS) and then fixed with 50% (v/v) acetone/methanol. After washing the fixed cells twice with PBS, the cells were then blocked with 3% Bovine Serum Albumen (BSA) for one hour and then a 1/100 dilution of the PRRSV monoclonal antibody SR30 (a generous gift from E.A. Nelson, Dept. Of Veterinary Science, South Dakota State University, Bookings, SD) in 0.01 M PBS, 0.05% Tween 20 and 2.95% NaCl was added to the cells. Following an incubation period of 1.5 hours, the cells were washed thrice with with 0.01 M PBS and 0.05% Tween 20. An alkaline phosphatase conjugated anti-mouse antibody (sold by Promega, Madison, WI) diluted 1/1000 in 0.01 M PBS, 0.05% Tween 20 and 2.95% NaCL was added and allowed to react for 1 hour. After

washing the cells thrice with 0.01 M PBS and 0.05% Tween 20, the cells were visualized by reacting with the chromogens, Nitro Blue Tetrazolium (NBT) and 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP) (both from Sigma,, St. Louis, MO) according to their recommendations. Cells were photographed on a Nikon Diaphot camera. Figure 7 shows that cells infected with the present invention reacted with the SR30 monoclonal anibodies.

Example 6

Killed Vaccine Production

The present invention is used to produce killed vaccines that are used to protect sows and gilts against PRRS infection. The antigenic mass of the present invention is used for vaccines. Once the invention is propagated to high titers, it would be readily apparent by those skilled in the art that the virus antigenic mass could be obtained by methods practiced by those skilled in the art. For example, the virus antigenic mass may be obtained by dilution, concentration, or extraction. All of these methods have been employed to obtain virus antigenic mass to produce vaccines.

The following is a description by which an inactivated vaccine is produced. The invention is grown according to a titer of 10^5 $TCID_{50}$/ml. The invention is then isolated by art-known methods and inactivated by treatment with formalin or with binary ethyleneimine (BEI), both methods are well known to those skilled in the art. The aforementioned inactivated virus of the present invention is mixed with any of the art-known adjuvants. The resulting vaccine formulations are administered to swine by methods familiar to those skilled in the art.

An example of inactivation by formalin is mixing the virus suspension of the present invention with 37 % formaldehyde to a final formaldehyde concentration of 0.05%. The virus-formaldehyde mixture is mixed by constant stirring for approximately 24 hours at room

temperature. The inactivated virus mixture is tested for residual live virus by assaying for growth on M-CSF treated monocytes of the present invention.

An example of inactivation by BEI is mixing the virus suspension of the present invention with 0.1 M BEI (2-bromo-ethylamine in 0.175 N NaOH) to a final BEI concentration of 1 mM. The virus-BEI mixture is mixed by constant stirring for approximately 48 hours at room temperature, followed by the addition of 1.0 M sodium thiosulfate to a final concentration of 0.1 mM. Mixing is continued for an additional two hours. The inactivated virus mixture is tested for residual live virus by assaying for growth on M-CSF treated monocytes of the present invention.

Example 7

Detection of Recombinant Virus in Host Cells

Blood, semen, or nasal swabs from swine inoculated with recombinant PRRSV containing ORF 1a and 1b of EAV and ORFs 2-7 of PRRSV are analyzed by RT-PCR. The cells contained within the above samples are lysed with Trizol Reagent (GIBCO-BRL, Gaithersburg, MD) according to the manufacturer's directions and as described in Example 1 and the RNA isolated. At least 1 to 5 μg of the isolated RNA is reversed transcribed into cDNA using Superscript II (GIBCO-BRL, Gaithersburg, MD) reverse transcriptase according to the manufacturer's direction and as described in Example 1. The cDNA from the reverse transcription reaction is PCR amplified using primers EAV-7 (SEQ ID NO: 7) and EAV-8 (SEQ ID NO: 8) using the following conditions. The PCR reaction volume is 100 μl and consists of 5 μl to 20 μl of the above mentioned cDNA reaction mixture, 1 μM each primer, 0.2 mM dNTP, 20 mM Tris-HCl pH 8.8, 10 mM KCL, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% (v/v) Triton-X-100, 100 μg/ml (w/v) bovine serum albumen, and 1 unit Taq Plus DNA polymerase (Stratagene, La Jolla, CA). The PCR reaction is performed in a GeneAmp 2400 thermal cycler (Perkin Elmer-Cetus, Norwalk, CT) under the following conditions: 94°C for 4

minutes, then 40 cycles consisting of 93°C for 45 seconds, 50°C for 30 seconds, and 72°C for 1 minute, followed by a 15 minute incubation at 72°C. After thermocycling approximately 20 µl of the reaction mixture is run on a 0.7% agarose gel an the band visualized by ethidium bromide intercalation. Primers EAV-7 and EAV-8 produce a 682 bp DNA product which is visualized on the agarose gel if the recombinant PRRSV of the present invention is present in the infected host cell.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: Stephen J. Spatz
Paul M. Coussens
John David Reilly

(ii) TITLE OF INVENTION: Recombinant Porcine reproductive and Respiratory Syndrome Viruses for Use as Vaccines and Diagnostics

(iii) NUMBER OF SEQUENCES: 26

(iv) CORRESPONDENCE ADDRESS:
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(B) STREET: 300 South Wacker Drive, 32nd Floor
(C) CITY: Chicago
(D) STATE: Illinois
(E) COUNTRY: USA
(F) ZIP: 60606

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: Windows 95
(D) SOFTWARE: Microsoft Word for Windows 97

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Smith, G. Kenneth
(B) REGISTRATION NUMBER: P43,135
(C) REFERENCE/DOCKET NUMBER: 97,245

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 312-913-0001
(B) TELEFAX: 312-913-90002

(2) INFORMATION FOR SEQ ID.:1

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.:1

GTTGCTAGCC ATATACGGCT CACCACCATA TACACTGC 38

(2) INFORMATION FOR SEQ ID.:2

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 23
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.:2

CATGGTCGAC AACGGTCACA CCG 23

(2) INFORMATION FOR SEQ ID.:3

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 26
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.:3

CGTTGTCGAC CATGCTCTTT ACAACC 26

(2) INFORMATION FOR SEQ ID.:4

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 23
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.:4

GTCTCTAGAG TCAGCAAAGG TCC 23

(2) INFORMATION FOR SEQ ID.:5

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.:5

GACTCTAGAG ACAAGGGTTT CG 22

(2) INFORMATION FOR SEQ ID.:6

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 26
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.:6

AAAGATGAAG TGGGATCCTC CCACGG 26

(2) INFORMATION FOR SEQ ID.:7

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.:7

GGAGGATCCC ACTTCATCTT TTCC 24

(2) INFORMATION FOR SEQ ID.:8

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 30
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.:8

TGCCTCGAGC ACAGAAATAG CAAAATCAGC 30

(2) INFORMATION FOR SEQ ID.:9

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 37
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.:9

GGCCTCGAGT GAAATGAAAT GGGGTCCATG CAAAGCC 37

(2) INFORMATION FOR SEQ ID.:10

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 46
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.:10

GCGAATTCAA TTTCGGCCGC ATGGTTCTCG CCAATTAAAT CTCACC 46

(2) INFORMATION FOR SEQ ID.:11

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.:11

CTAGAGATAT CGAATTCGGT AC 22

(2) INFORMATION FOR SEQ ID.:12

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 14
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.:12

CGAATTCGAT ATCT 14

(2) INFORMATION FOR SEQ ID.:13

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 36
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.:13

CTCTAGACTG CAGGGATCC TCGAGGAATT CGGTAC 36

(2) INFORMATION FOR SEQ ID.:14

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 36
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.:14

CTCTAGACTG CAGGGATCCC TCGAGGAATT CGGTAC 36

(2) INFORMATION FOR SEQ ID.:15

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 51
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.:15

CTAGTAATAC GACTCACTAT AGGCTAGCTC TAGAGATATC GAATTCGGTA C 51

(2) INFORMATION FOR SEQ ID.:16

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 42
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.:16

CGAATTCGAT ATCTCTAGAG CTAGCCTATAG TGAGTCGTAT TA 42

(2) INFORMATION FOR SEQ ID.:17

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 50
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.:17

CTAGATTTAG GTGACACTAT AGCTAGCTTA GAGATATCGA ATTCGGTAC 50

(2) INFORMATION FOR SEQ ID.:18

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 43
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.:18

CGAATTCGAT ATCTCTAGAG CTAGCCTATA GTGAGTCGTA TTA 43

(2) INFORMATION FOR SEQ ID.:19

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 72
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.:19

GTTGCTAGCA TTTAGGTGAC ACTATAGCTC GAAGTGTGTA TGGTGCCATA TACGGCTCAC 60

CATATACACT GC 72

(2) INFORMATION FOR SEQ ID.:20

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 74
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.:20

GTTGCTAGCT AATACGACTC ACTATAGCTC GAAGTGTGTA TGGTGCCATA TACGGCTCAC 60

CACCATATACA CTGC 74

(2) INFORMATION FOR SEQ ID.:21

(i) SEQUENCE CHARACTERISTICS
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.:21

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CACCATATAC ACTGC 75

(2) INFORMATION FOR SEQ ID.:22

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 75
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.:22

GCCCATATGT AATACCAGTC ACTATAGCTC GAAGTGTGTA TGGTGCCATA TACGGCTCAC 60

CACCATATAC ACTGC 75

(2) INFORMATION FOR SEQ ID.:23

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.:23

GTTTCAGCGG AACAATGG 18

(2) INFORMATION FOR SEQ ID.:24

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 19
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.:24

CCCTAATTGA ATAGGTGAC 19

(2) INFORMATION FOR SEQ ID.:25

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.:25

GTCTGGATTG ACGACAGACA 20

(2) INFORMATION FOR SEQ ID.:26

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.:26

AACCACGCAT TTGTCGTCCG 20

CLAIMS

What is claimed:

1. A nucleic acid encoding a polymerase from an RNA virus, excluding PRRSV, and ORFs 2-7 of PRRSV.

2. A nucleic acid encoding a polymerase from a virus from the *Coronavirus* family, excluding PRRSV, and ORFs 2-7 of PRRSV.

3. A nucleic acid encoding a polymerase from a virus from the *Arteritis* genus, excluding PRRSV, and ORFs 2-7 of PRRSV.

4. The nucleic acid of claims 1, 2, or 3, wherein the nucleic acid is RNA.

5. The nucleic acid of claims 1, 2 or 3, wherein the nucleic acid is DNA.

6. A nucleic acid encoding ORF 1a and ORF 1b of EAV and ORFs 2-7 of PRRSV.

7. The nucleic acid of claim 6, further comprising the 5' untranslated region of EAV.

8. The nucleic acid of claim 6, further comprising the 3' untranslated region of PRRSV.

9. The nucleic acid of claim 6, further comprising the 5' untranslated region of PRRSV.

10. The nucleic acid of claim 7, further comprising the 3' untranslated region of PRRSV.

11. The nucleic acid of claim 10, wherein the 5' untranslated region of EAV is 5' to the ORF 1a and 1b of EAV which is 5' to the ORFs 2-7 of PRRSV which is 5' to the 3' untranslated region of PRRSV.

12. The nucleic acid of claim 8 further comprising the 5' untranslated region of PRRSV.

13. The nucleic acid of claim 12, wherein the 5' untranslated region of PRRSV is 5' to the ORF 1a and ORF 1b of EAV which is 5' to the ORFs 2-7 of PRRSV which is 5' to the 3' untranslated region of PRRSV.

14. The nucleic acid of claim 9, further comprising the 3' untranslated region of EAV.

15. The nucleic acid of claim 14, wherein the 5' untranslated region of PRRSV is 5' to the ORF 1a and ORF 1b of EAV which is 5' to the ORFs 2-7 of PRRSV which is 5' to the 3' untranslated region of EAV.

16. The nucleic acid of claim 11, further comprising a viral RNA promoter.

17. The nucleic acid of claim 13, further comprising a viral RNA promoter.

18. The nucleic acid of claim 15, further comprising a viral RNA promoter.

19. The nucleic acid of claim 16 wherein the viral RNA promoter is selected from the group consisting of cytomegalovirus promoter, herpes simplex thymidine kinase promoter, SV40 promoter or rous sarcoma long terminal repeat promoter.

20. The nucleic acid of claim 17 wherein the viral RNA promoter is selected from the group consisting of cytomegalovirus promoter, herpes simplex thymidine kinase promoter, SV40 promoter or rous sarcoma long terminal repeat promoter.

21. The nucleic acid of claim 18 wherein the viral RNA promoter is selected from the group consisting of cytomegalovirus promoter, herpes simplex thymidine kinase promoter, SV40 promoter or rous sarcoma long terminal repeat promoter.

22. The nucleic acid of claim 16, further comprising a bacteriophage RNA polymerase promoter.

23. The nucleic acid of claim 17, further comprising a bacteriophage RNA polymerase promoter.

24. The nucleic acid of claim 18, further comprising a bacteriophage RNA polymerase promoter.

25. The nucleic acid of claim 22 wherein the bacteriophage RNA polymerase promoter is selected from the group consisting of T3, T7, or Sp6.

26. The nucleic acid of claim 23 wherein the bacteriophage RNA polymerase promoter is selected from the group consisting of T3, T7, or Sp6.

27. The nucleic acid of claim 24 wherein the bacteriophage RNA polymerase promoter is selected from the group consisting of T3, T7, or Sp6.

28. A plasmid comprising the nucleic acid of claim 19, 20, 21, 25, 26, or 27 wherein a viral RNA promoter is operationally linked to EAV ORFs 1a and 1b and PRRSV ORFs 2-7.

29. A plasmid comprising the nucleic acid of claim 25, 26, or 27 wherein a bacteriophage RNA polymerase promoter is operationally linked to EAV ORFs 1a and 1b and PRRSV ORFs 2-7.

30. The plasmid pEP.

31. The plasmid pEP-T7.

32. The plasmid pEP-T3.

33. The plasmid pEP-Sp6.

34. The plasmid of claim 29 wherein the plasmid is transcribed *in vitro* to make an RNA transcript which is transfected into a host cell to produce an infectious recombinant PRRSV.

35. A host cell containing the nucleic acid of claims 28 wherein the nucleic acid is transcribed within the host cell to make an infectious recombinant PRRSV.

36. A host cell containing the plasmid of claim 29 wherein the plasmid is transcribed within the host cell to make an infectious recombinant PRRSV.

37. The host cell of claim 34, 35, or 36 wherein the host cell is a porcine macrophage.

38. The host cell of claim 34 or 35, or 36 wherein the host cell is a porcine monocyte.

39. The host cell of claim 34 or 35, or 36 wherein the host cell is a simian derived cell line.

40. A recombinant PRRSV comprising the nucleic acid of claim 1, 2, 3, 11, 13, or 15.

41. A vaccine against PRRSV comprising the recombinant virus from claim 40.

42. The vaccine of claim 41, further comprising a physiological carrier.

43. The vaccine of claim 41, further comprising an adjuvant.

44. A method of making a killed vaccine comprising:

(a) isolation of the recombinant virus of claim 40

(b) chemically inactivating the virus

(c) adding a physiological carrier.

45. A method of making a live vaccine comprising:

(a) isolation of the recombinant virus of claim 40

(b) chemically inactivating the virus

(c) adding an adjuvant.

46. A method for detecting a recombinant PRRSV which contains a marker comprising:

(a) isolating the virus from infected cells;

(b) isolating nucleic acid from the virus;

(c) making a cDNA copy of the virus

(c) performing PCR on the cDNA using two primers complementary to the marker; and

(d) detecting a PCR product.

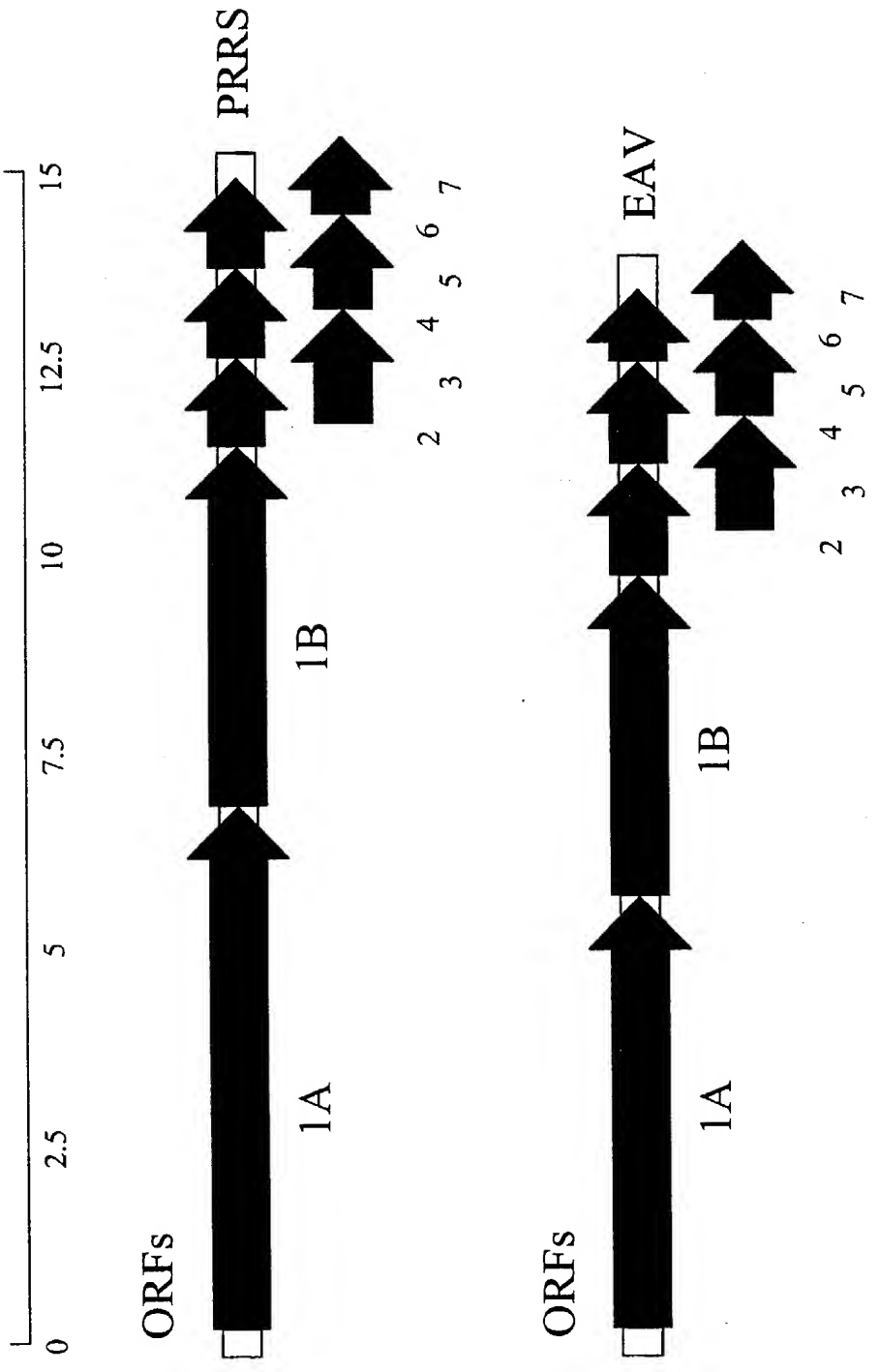


Fig. 1

Fig. 2

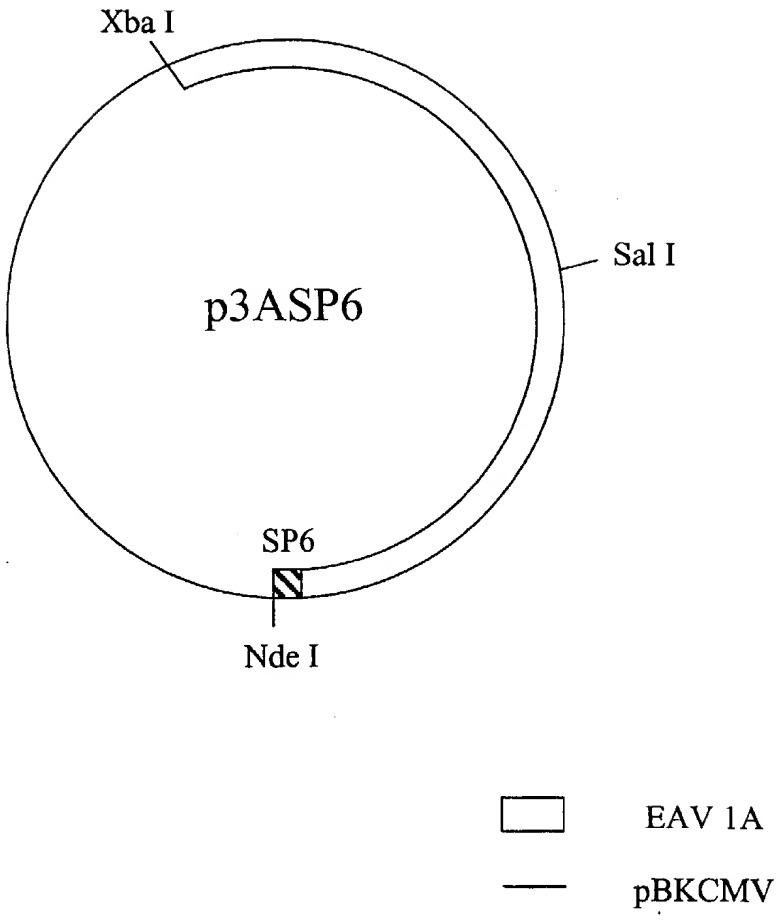


Fig. 3A

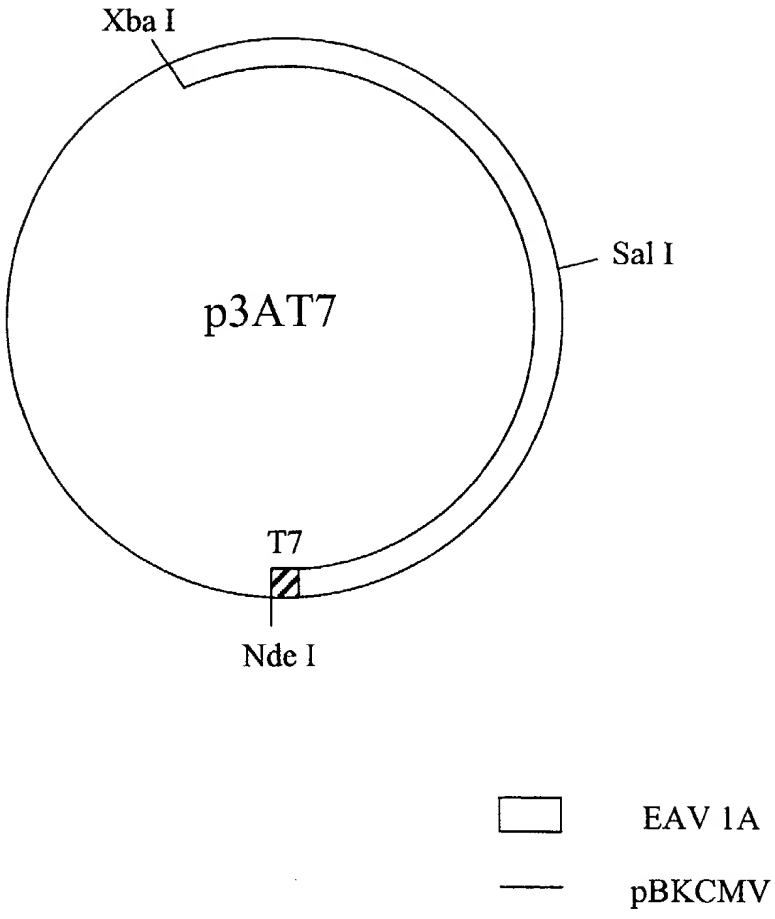
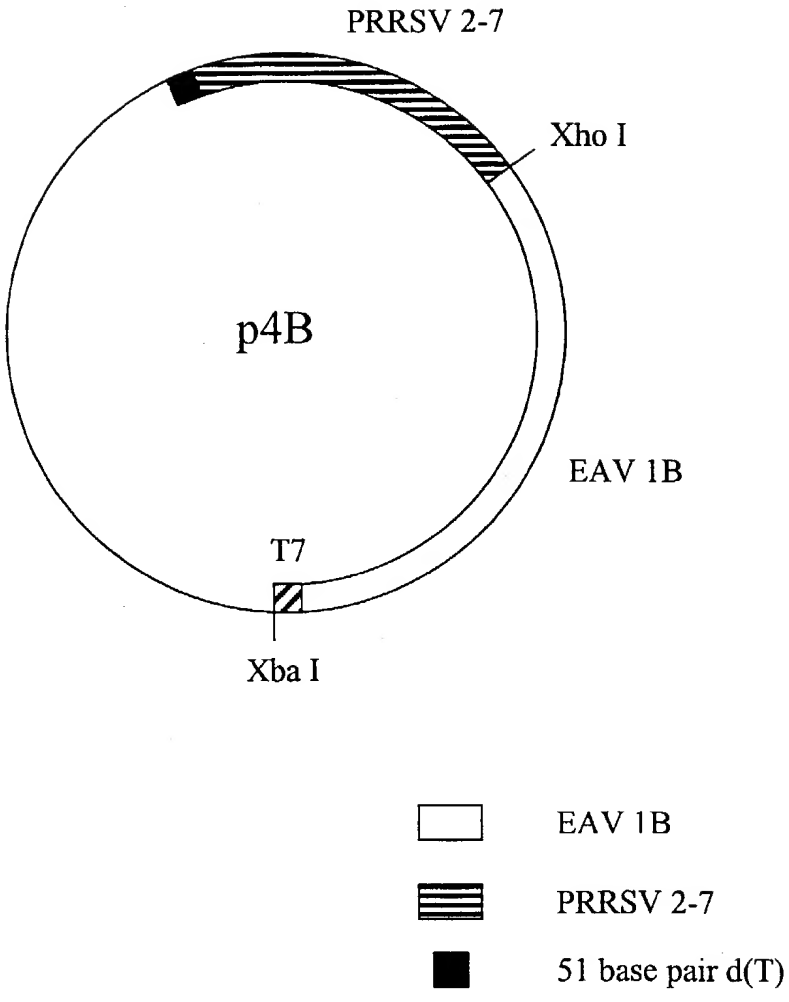


Fig. 3B



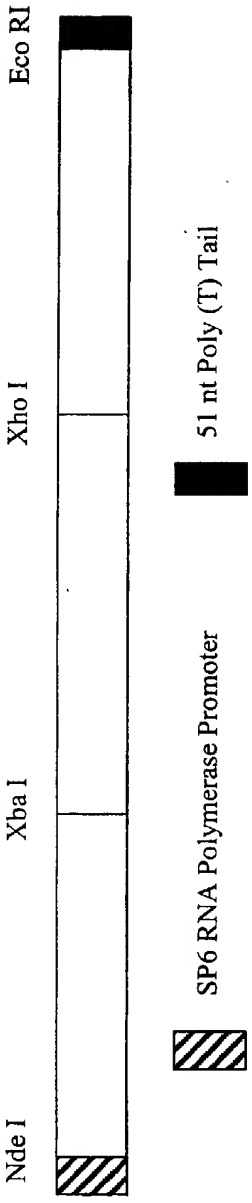

Nde I
Xba I
Xho I
Eco RI
SP6 RNA Polymerase Promoter
51 nt Poly (T) Tail

Fig. 4

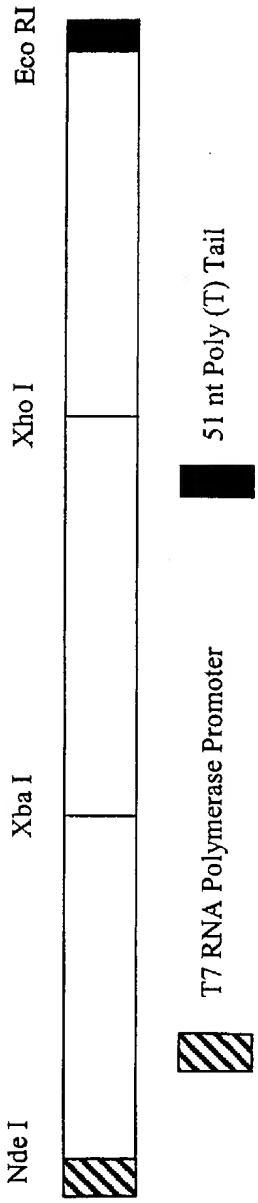

Nde I
Xba I
Xho I
Eco RI
T7 RNA Polymerase Promoter
51 nt Poly (T) Tail

Fig. 5

Fig. 6

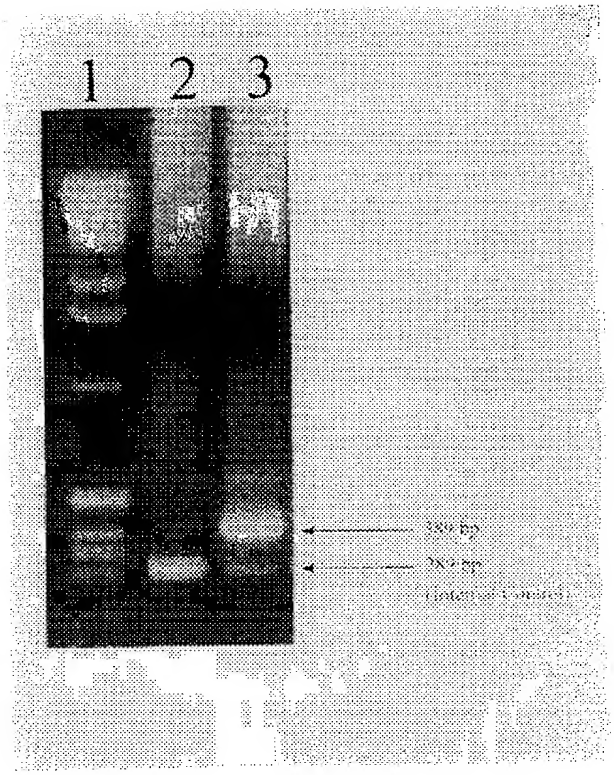
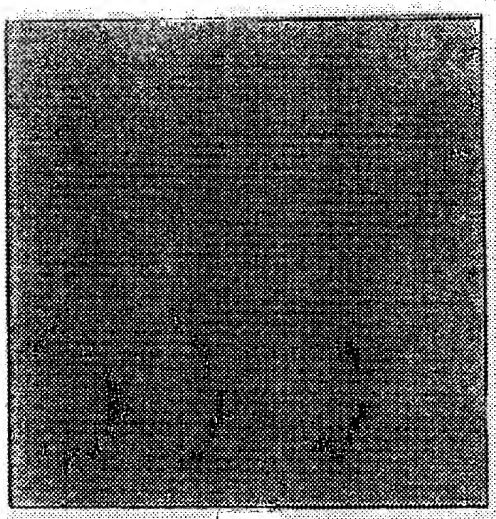
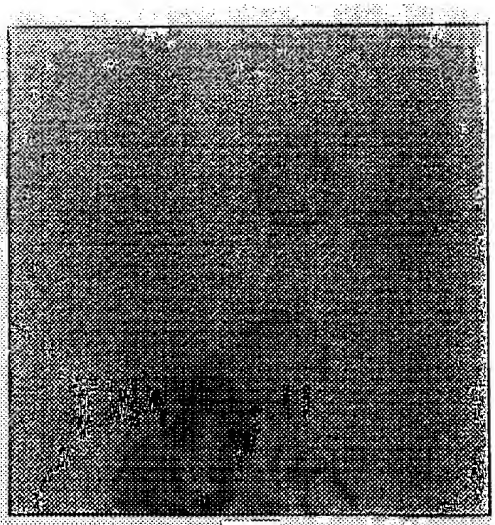


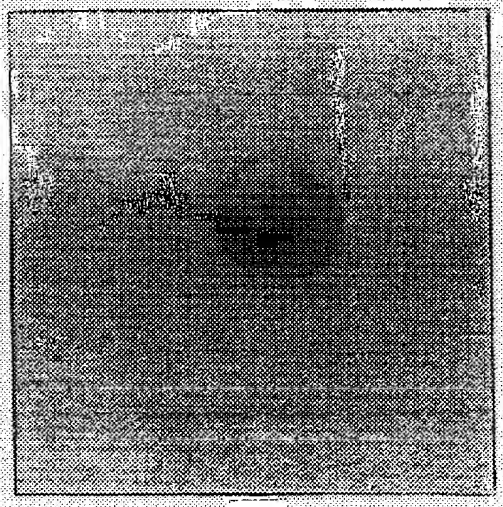
Fig. 7



7A



7C



7B